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# Maternal antibody transmission and breeding densities in the Black-headed Gull *Larus ridibundus*

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## Summary

1. The offspring of avian species, especially those of colonial breeders, are exposed to a number of pathogens immediately after birth. The chick's immune system is, at that early stage still immature and inefficient. As a consequence, diseases can have a strong impact on chick survival.
2. The ability of mothers to transmit passive immunity in terms of antibodies of their own acquired immunity to their chicks is probably an essential pathway to enhance the chick survival. Since the production of antibodies is costly, females are expected to adjust the transmission of passive immunity to the local disease environment.
3. We found that in Black-headed Gulls (*Larus ridibundus* L.) yolk antibody concentrations are positively correlated with local breeding density. This transmission pattern is likely to be adaptive, as the aggregation of birds will enhance the local presence of pathogens.
4. When birds were forced to re-settle after the colony had been flooded (10–20% of the original number of breeding pairs re-settled), this relationship was no longer present. The lack of such a relationship may be explained by the fact that females may retain certain levels of antibodies as a consequence of infections that occurred during the first breeding attempt at a different breeding density.
5. Within clutches, maternal antibodies decreased with laying sequence, in particular in eggs hatching male chicks. This transmission pattern may contribute to the observed mortality pattern found in gull species.

**Key-words:** colonial breeding, laying order, maternal effects, passive immunity.

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## Introduction

In colonial breeding species, individuals can benefit from living in groups since it improves their defence against predators and their foraging efficiency (e.g. Ward & Zahavi 1973; Götemark & Andersson 1985; Danchin & Wagner 1997). Coloniality also entails costs. The aggregation of large numbers of individuals during the breeding season enhances the infection risks including a greater risk of acquiring parasites (e.g. Brown & Brown 1986; Tella 2002). In colonial breeding species, this relationship not only applies to adults but also to their chicks that are faced with pathogens immediately after hatching. Therefore, early immune function might be important to guarantee subsequent survival (e.g. Christe, Möller & de Lope 1998; Müller, Dijkstra & Groothuis 2003). In contrast to adults that possess a fully developed immune system, chicks have initially an inefficient

immune system because the humoral immune response is not yet developed (Apanius 1998). To compensate for this, females can transfer components of their own acquired immunity to their offspring. These early maternal effects can adaptively influence the chick phenotype via non-genetic inheritance (Mousseau & Fox 1998).

Thus, maternal antibody transmission might be a specific way to modify a chick's phenotype to the local disease environment, which varies between nesting sites within a colony, according to the local breeding density.

Indeed, previous studies showed that females increase their antibody production when allocating maternal antibodies to their offspring, probably to sustain their own baseline levels while providing the eggs (Klasing 1998; Saino, Martinelli & Möller 2001). Both concentration and diversity of transferred antibodies correlate with the pathogen exposure of the female before egg laying (e.g. Gasparini *et al.* 2001). This suggests that an infection at the nesting site (Gasparini *et al.* 2001) or re-activation of a dormant infection might affect the antibody concentrations. In general, females cannot transfer antibodies towards pathogens they have

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not been exposed to themselves (Heller *et al.* 1990; Leitner *et al.* 1990). The transfer of specific antibodies seems adaptive since it has been shown that it reduces the susceptibility of the offspring to those pathogens (reviewed in Grindstaff, Brodie & Ketterson 2003).

The transmission of maternal antibodies involves the price of mounting an immune response in the mother (reviewed by Sheldon & Verhulst 1996), so females are expected to adjust their transmission to the exposure to pathogens at the current nesting site. Therefore, they may face limitations in the transmission of antibodies for nutritional reasons (reviewed in Grindstaff *et al.* 2003). Their endocrine state may also hamper the antibody transmission due to potentially suppressive effects of androgens on the acquired immune system (e.g. Grossman 1985). This especially applies to species such as Black-headed Gulls *Larus ridibundus*. As in other bird species of the temperate zone, females increase their androgen levels with the onset of the breeding season (Wingfield *et al.* 1990; Groothuis & Meeuwissen 1992). In these gulls, both sexes show territorial behaviour and an increase in breeding density will lead to a higher number of social/territorial interactions. It is well known that this stimulates testosterone in the bird, which is necessary to maintain the territory (Beletsky, Orians & Wingfield 1992; Silverin 1998; Alonso-Alvarez & Velando 2001). This may lead to a problem for the female because the need for antibody transmission increases as the breeding density increases, while at the same time her antibody production and so her transmission may be hampered due to her enhanced androgen levels.

In this study, we investigated levels of maternal antibodies in the eggs of black-headed gulls in relation to local breeding density. As our field site was completely flooded at the end of the incubation period, we were also able to analyse antibody concentrations of clutches that were laid when the colony re-settled with much lower breeding densities. This enables us to analyse the flexibility of the maternal antibody transmission pattern.

In the analysis we further included laying order and sex of the eggs, because both affect survival probabilities of the chicks in this species (Müller *et al.* 2003). Clearly, survival may be associated with immune function (Müller *et al.* 2003), and differential maternal transmission of immunity could be responsible for the variation in chick performance (Blount *et al.* 2001; Saino *et al.* 2003).

## Materials and methods

### STUDY SPECIES AND EGG COLLECTION

Black-headed Gulls are monogamous, colonial breeders. Within the breeding colonies, they defend small territories, starting about 2 weeks before egg laying takes place. The modal clutch is three eggs that are laid over a 3- to 5-day period (Cramp & Simmons 1983). In 2001, a large black-headed gull colony (about 2000 breeding pairs distributed over several subcolonies) along the north-east coast of the Netherlands was checked daily

for nest building and freshly laid eggs. These eggs were marked with a non-toxic marker referring to the position in the laying order and the date of laying. On the day of clutch completion, all eggs from a total of 30 clutches were collected, weighed to the nearest 0.1 g and placed in an incubator at 37.5 °C with 60% humidity to allow some embryonic development for sex determination. As some incubation takes place prior to clutch completion, the eggs were incubated differentially to approximately equalize the total incubation time (60 h in the case of the first laid egg, 72 h for the second-laid egg and 84 h for the last-laid egg). All eggs have hence been incubated less than 7 days in total, to guarantee that transport of antibodies into the embryo did not change the antibody concentrations as measured in the yolk (Kowalczyk *et al.* 1985).

We collected clutches during the normal laying period in May–June ( $n = 16$ ) and again in late July ( $n = 14$ ) after the whole colony area had been flooded in a western storm. The late ones were probably replacement clutches.

At the time the clutch was collected, we counted the number of nests of the same species (= neighbours) within a radius of 3 m around the nest as an indicator of density. The radius of 3 m approximates (depending on the vegetation cover) the distance chicks move away from their nest during the first 2 weeks of life.

### MOLECULAR SEX DETERMINATION

The eggs collected were defrosted and the yolk and embryo separated. A small tissue sample of the embryo was used for Chelex® resin-based DNA extraction (Walsh, Metzger & Higuchi 1991). 2 µl of the resulting DNA solution was used in a polymerase chain reaction (PCR) to amplify a part of the CHD-W gene in females and the CHD-Z gene in both sexes (for details see Griffiths, Orr & Dawson 1998). The reliability of this method has been established in earlier studies on this species (e.g. Müller *et al.* 2003).

### ENZYME-LINKED IMMUNO-SORBENT ASSAY FOR ANTIBODY DETERMINATION

Antibody concentrations were determined using an indirect Enzyme-Linked Immuno-Sorbent Assay (ELISA) with commercial anti-chicken antibodies (Sigma® C-6409). This method provides a sensitive measurement of antibody concentrations that bind to a specific antigen (Janeway & Travers 1999). Using commercial anti-chicken antibodies in a direct ELISA has been shown to be a reliable technique to determine antibody concentrations for a variety of bird species (Martinez *et al.* 2003). In addition, we validated the assay for yolk samples of our study species. Briefly, samples were separated by means of SDS-Page under reducing conditions; the gels had been Western blotted onto nitrocellulose filters and immune stained with the anti-chicken antibody used in the ELISA. The results show that the antibody detects only the light chain of the immunoglobulins (molecular weight 22 kDa) without binding

to the heavy chain of the immunoglobulins or any other protein. Thus, using indirect ELISA is well suited for measuring yolk antibodies of black-headed gulls.

The yolk was diluted 1 : 1 with distilled water; 2 mg of the sample was taken and pH adjusted to 5.0–5.2 with 1 M HCl. After centrifugation at 10 000 *g* for 25 min, the supernatant containing the antibodies was separated and the antibody concentrations were determined using indirect ELISA. To assess total antibody levels, a standard of pooled yolk of all individuals included in the analysis was used, which was assigned a concentration of 10<sup>6</sup> arbitrary. All values were subsequently expressed relative to this standard (Antibody index = relative antibody titer/10 000). Briefly, 96-well ELISA plates (Nunc<sup>TM</sup> Immunoplate) were coated with anti-chicken antibodies (concentration 1 : 180, Sigma<sup>®</sup> C-6409) by overnight incubation at 4 °C. After emptying the wells, they were incubated with 1% BSA- PBS (Roche Diagnostics<sup>®</sup>, Espoo, Finland) for 1 h, then washed with 300 µl PBS-Tween (three times). Yolk samples were diluted 1 : 10 000 in 1% BSA-PBS to ensure that the samples are on the linear part of the standard curve. 50 µl of this dilution was added in replicate to the wells. Likewise dilution series of the standard was added in replicate, including a negative control. The plates were incubated for 3 h at room temperature, and then washed with 300 µl PBS-Tween (three times). Subsequently, an alkaline phosphatase conjugated secondary antibody (Sigma<sup>®</sup> A-9171 anti-chicken IgG, 1 : 15 000 diluted in 1% BSA-PBS) was added. Plates were incubated for another night at 4 °C. In the last step plates were washed with 400 µl PBS-Tween (three times) and 100 µl of an alkaline phosphatase substrate (PNPP, p-nitro phenol phosphate, Sigma<sup>®</sup> 104 phosphatase substrate in 1 M diethanol amine buffer [1 mg ml<sup>-1</sup>]) was applied (50 µl).

The absorbance was measured at 405 nm in an ELISA reader 45 min after adding the substrate (or until the lowest standard reached the absorbance of 2.0).

#### STATISTICAL PROCEDURES

All data were analysed in a backward elimination procedure using hierarchical linear models in the MLwiN program 1.10 (Rasbash *et al.* 2000). This method accommodates unbalanced data, allows analyses of variance and covariance taking into account the nested relationship of different eggs in a nest and controls for multiple (independent) variables. Significance was tested using the increase in deviance ( $\Delta$  deviance) when a factor was removed from the model, which follows a  $\chi^2$ -distribution (Wald statistic). The following variables were included in the analysis: number of neighbours (density), egg mass, laying order, laying date, sex of the embryo and all possible interactions. When analysing the contribution of egg mass and offspring sex, we were mainly interested in the within-nest effects, hence mean egg mass and mean sex ratio of the clutch were also included in the model (Snijders & Bosker 1999). Post hoc tests were done using the same program.

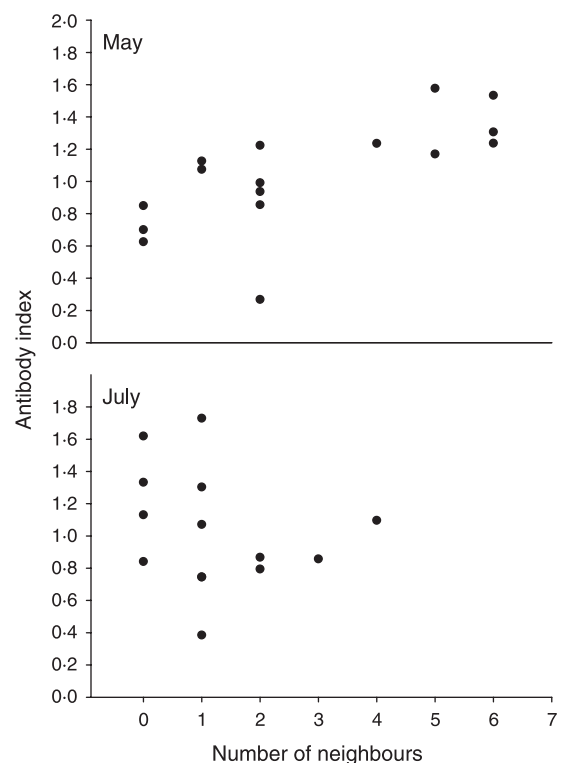
Because our data consists of two sets – early clutches ( $n = 16$ ) laid during the ‘normal’ breeding season; late clutches ( $n = 14$ ) laid after the whole colony area had been flooded – we analysed each set separately.

#### Results

Within the early clutches ( $n = 16$ ), antibody concentrations of a clutch positively correlated with the number of neighbours within a radius of 3 m (Fig. 1, Number of neighbours: estimate 0.10, error 0.03,  $\Delta$  deviance = 9.90, d.f. = 1,  $P = 0.002$ ).

Antibody concentrations showed variation both between and within clutches. Notably, antibody concentrations of a particular egg within a clutch decreased through the laying sequence (laying sequence:  $\Delta$  deviance 6.08, d.f. = 2,  $P < 0.05$ ), with first eggs having the highest antibody concentrations.

In the second model, we analysed the clutches laid during the second breeding attempt in July ( $n = 14$ ). In contrast to the early clutches, there was no effect of the number of neighbours on the transmission of antibodies (Fig. 1, Number of neighbours: estimate -0.10, error 0.13,  $\Delta$  deviance = 0.30, d.f. = 1,  $P = 0.58$ ). Within clutches, antibody titres varied with the position in the laying sequence in interaction with embryonic sex (sex laying sequence:  $\Delta$  deviance = 6.81, d.f. = 2,  $P = 0.03$ ). This is due to the fact that antibody concentrations



**Fig. 1.** Yolk antibody concentrations (mean value of each clutch [antibody index = relative antibody concentration, see Methods]) in relation to the breeding density, measured as number of neighbours within the radius of 3 m (May) during the normal breeding season (July) after a period of re-laying at the end of the breeding season.

were highest in first-laid eggs in males, but did not vary with laying sequence in females (post hoc males ( $n = 24$ ), laying order:  $\Delta$  deviance = 10.39, d.f. = 2,  $P = 0.005$ ; post hoc females ( $n = 18$ ), laying order:  $\Delta$  deviance = 1.96, d.f. = 2,  $P = 0.38$ ). Male antibody titres were significantly higher compared to female eggs in first laid eggs ( $\Delta$  deviance = 5.44, d.f. = 1,  $P = 0.02$ ), but significantly lower in second laid eggs ( $\Delta$  deviance = 6.89, d.f. = 1,  $P = 0.01$ ). There were no sex differences in yolk antibody concentrations in last laid eggs ( $\Delta$  deviance = 0.06, d.f. = 1,  $P = 0.80$ ).

## Discussion

### BETWEEN-CLUTCH VARIATION

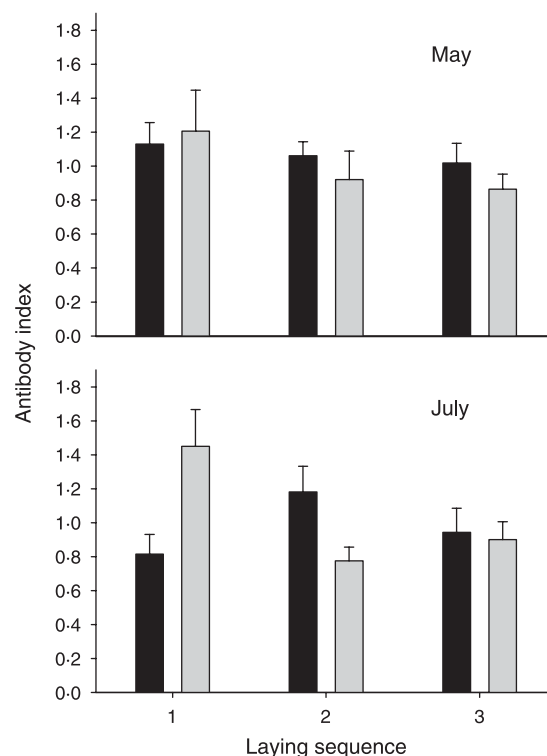
We found that the transmission of maternal yolk antibodies correlated with the number of breeding pairs within a radius of 3 m around the nest. This transmission pattern is likely to be an adaptive maternal investment. The transmission of high levels of passive immunity via the mother has been shown to enhance disease resistance of the chick (reviewed in Grindstaff *et al.* 2003). Since the local pathogen exposure is likely to be relatively high at nest sites with high breeding density, the transmission of antibodies might be more beneficial than elsewhere. These data are consistent with the transmission pattern in a seabird–ectoparasite system (Kittiwake *Rissa tridactyla* – seabird ticks *Ixodes uriae*) studied by Gasparini *et al.* (2001). These authors found that when the local ectoparasite density was high, the yolk antibody concentrations against disease transmitted by these ectoparasites were also enhanced. In their study system, actual breeding density and local ectoparasite concentration were not associated. Ticks over-winter at the host nest site and can accumulate according to the occupancy of the nest site of the birds (Gasparini *et al.* 2001).

In contrast, due to the frequent flooding of the breeding site in autumn and winter, pathogen exposure in Black-headed Gulls depends on the actual number of birds present during that breeding season, and thus very likely increases with density. It is not a function of densities in previous years as in the Kittiwake system. At dense breeding areas, elevated levels of aggressive encounters will lead to higher plasma testosterone levels (e.g. Beletsky *et al.* 1992). In our study species, both sexes are involved in establishment and maintenance of the territory. Thus, females also have elevated plasma testosterone levels, and are similarly affected by the relationship between social environment and endocrine state (Wingfield *et al.* 1990; Groothuis & Meeuwissen 1992). We therefore hypothesized that females may be limited in their transmission of passive immunity, especially if breeding density is high, because high maternal androgen levels resulting from high breeding densities may lead to immunosuppression (e.g. Grossman 1985). We did not find any evidence for this as females had no reduced transmission of antibodies to their eggs in

areas with high density. However, further experimental studies are needed to examine this relationship.

Alternatively, the antibody pattern we found may also be explained by the age structure in a colony. As in a number of other colonial seabird species, young birds tend to breed at the outer boundaries of a colony with lower local breeding density (e.g. Wooller & Coulson 1977; Montevecchi 1978). Thus, the increase of maternal yolk antibodies may partly reflect maternal age, as females cannot transfer antibodies towards pathogens they have not been exposed to themselves (Heller *et al.* 1990; Leitner *et al.* 1990). However, this is not a likely explanation because the diversity of antibodies transmitted may vary according to age, but the total maternal antibody titres did not correlate with age in Common Terns *Sterna hirundo* (Apanius & Nisbet 2003).

The relationship between maternal antibody transmission and breeding density was only present during the initial phase of colony establishment. When the first chicks had just hatched (about 4–5 weeks after first egg laying), the whole colony area was flooded. Two weeks later, about 10–20% of the birds had re-settled and started a replacement clutch. The range in clutch levels of maternal yolk antibodies was not reduced during the re-laying period (Fig. 1) although, at the time of re-laying, pathogen exposure was probably reduced, given the long-lasting flood of salt water.



**Fig. 2.** Yolk antibody concentrations [antibody index = relative antibody concentration, see Methods] in relation to the position within the laying sequence and embryonic sex [males (grey bars), females (black bars); mean  $\pm$  SE] (May) during the normal breeding season (July) after a period of re-laying at the end of the breeding season.



This may be explained by the possibility that females may have enhanced their plasma antibody concentrations according to the pathogen exposure at the nest site during the first breeding attempt. After the infection, antibodies then remain high for weeks post-infection (Ots & Hõrak 1998).

After the reduction in numbers of breeding pairs, there was no longer a defined colony structure. It is likely that pairs returned to or stayed at their previous territory. After resettlement, the transmission pattern of antibody titres was no longer related to the actual breeding density, because the time span between change in pathogen exposure and re-laying was probably too short for the females to adjust their transmission pattern. Alternatively, if pairs moved to a new territory, lateness of the year may have forced them to take quick decisions where to breed, without allowing for an adjustment of the transmission pattern to nest site characteristics.

#### WITHIN CLUTCH VARIATION

Yolk antibody concentrations decreased with laying order, as has been shown for the Lesser Black-backed Gull *Larus fuscus* (Blount 2001). Reduced antioxidant reserves (Royle *et al.* 1999, Blount *et al.* 2001), smaller size (e.g. Royle *et al.* 1999) and low passive immunity (this study, Blount *et al.* 2001), and delayed hatching together may contribute to the reduced probability of survival of the last chick (Müller *et al.* 2003). This decrease of egg and consequently chick quality is particularly marked in gull species, and might also indicate difficulties for the bird in maintaining egg quality during the laying period.

When re-laying, the decrease in antibody concentrations with laying sequence was only present in eggs hatching male chicks. These data may indicate that females adjust the amount of antibodies and the sex of the egg towards each other (Saino *et al.* 2003). From a mechanistic point of view, sex-specific differences in the transmission pattern with laying order are difficult to explain, due to the lack of indications of active transport of antibodies into the yolk (reviewed in Grindstaff *et al.* 2003).

However, sex-specific transmission pattern may be causally linked to the male-biased mortality that frequently has been reported for gull species (Griffiths 1992; Nager *et al.* 2000). Depending on the time of the year, females may follow different sex-specific transmission strategies within the laying sequence, according to the probability of rearing the more vulnerable sex. Further studies are needed to evaluate the adaptive significance of the observed sex differences in yolk antibody concentrations.

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